PART I - ADMINISTRATIVE

Section 1. General administrative information

Title of project

Viral Vaccines And Effects On Reproductive Status

BPA project number: 20048
Contract renewal date (mm/yyyy):   ☐ **Multiple actions?**

Business name of agency, institution or organization requesting funding

Business acronym (if appropriate)   WSU

Proposal contact person or principal investigator:
Name	Sandra S. Ristow
Mailing Address	Dept. Animal Sciences, Washington State University
City, ST Zip	Pullman, WA 99164-6351
Phone	509-335-0165
Fax	509-335-4246
Email address	ristow@wsu.edu

NPPC Program Measure Number(s) which this project addresses
7.2, 7.4D, (1994 Columbia River Basin Fish and Wildlife Program)

FWS/NMFS Biological Opinion Number(s) which this project addresses

Other planning document references
Endangered Species Act Section 7a2

Short description
Develop a vaccine for IHNV and test its efficacy and effect on reproductive status of salmonids

Target species
Oncorhynchus mykiss (Steelhead and Rainbow trout)

Section 2. Sorting and evaluation

Subbasin
Systemwide

Evaluation Process Sort

CBFWA caucus	Special evaluation process	ISRP project type
Mark one or more caucus	If your project fits either of these processes, mark one or both	Mark one or more categories
☒ Anadromous fish	☒ Multi-year (milestone-based	☐ Watershed councils/model watersheds

☐ Resident fish	evaluation)	☐ Information dissemination
☐ Wildlife	☐ Watershed project evaluation	☐ Operation & maintenance
		☐ New construction
		☒ Research & monitoring
		☐ Implementation & management
		☐ Wildlife habitat acquisitions

Section 3. Relationships to other Bonneville projects

Umbrella / sub-proposal relationships. List umbrella project first.

Project #	Project title/description

Other dependent or critically-related projects

Project #	Project title/description	Nature of relationship
1	Intracytoplasmic Sperm Injection: Genetic Retrieval	Project Participant
2	Endocrine Control of Ovarian Development in Salmonids	Project Participant
3	Analyzing Genetic and Behavioral Changes During Salmonid Domestication	Project Participant
4	Induction of Precocious Sexual Maturity and Enhanced Egg Production in Fish	Project Participant
5	Enhancement of Salmonid Gamete Quality by Manipulation of Intracellular ATP	Project Participant

Section 4. Objectives, tasks and schedules

Past accomplishments

Year	Accomplishment	Met biological objectives?
	None	

Objectives and tasks

Obj 1,2,3	Objective	Task a,b,c	Task
1	Express the G and N proteins of IHNV on the surface of Yersinia ruckerii	a	Construct the G expression plasmid
		b	Express the N expression plasmid
2	Fix the recombinantly made Yesinias containing the G and N proteins with formalin or beta-propiolactone	a	Fix the recombinantly made Yesinias containing the G and N proteins with formalin or beta-propiolactone

Objective schedules and costs

Obj #	Start date mm/yyyy	End date mm/yyyy	Measureable biological objective(s)	Milestone	FY2000 Cost %
1	10/1999	10/2000	Express the G and N proteins of IHNV		.90%
2	9/2000	10/2000	Fix the recombinantly made Yesinias containing the G and N proteins with formalin or beta-propiolactone		.10%
				Total	1.00%

Schedule constraints
None

Completion date
10/2004

Section 5. Budget

FY99 project budget (BPA obligated): $0

FY2000 budget by line item

Item	Note	% of total	FY2000
Personnel	Sandra Ristow 1.5 Technicians, Student Timeslip	%30	61,586
Fringe benefits	Sandra Ristow 26%; Technicians 29%; Timeslip 9%	%8	17,330
Supplies, materials, non-expendable property	Molecular biology supplies, restriction enzymes; Flow cytometer, Supplies	%9	18,055
Operations & maintenance	Repair of equipment	%1	3,000
Capital acquisitions or improvements (e.g. land, buildings, major equip.)		%0	
NEPA costs		%0	
Construction-related support		%0	
PIT tags	# of tags:	%0	
Travel		%1	2,000
Indirect costs	@45% of Direct Costs (exclude equipment)	%27	54,887
Subcontractor		%0	
Other	Administrative Costs $5,000; Aquaculture Core $15,000; PCR $8,685; Documentation System $19,344	%23	48,029
TOTAL BPA FY2000 BUDGET REQUEST			$204,887

Cost sharing

Organization	Item or service provided	% total project cost (incl. BPA)	Amount ($)
		%0	
		%0	
		%0	
		%0	
	Total project cost (including BPA portion)		$204,887

Outyear costs

	FY2001	FY02	FY03	FY04
Total budget	$215,131	$225,888	$237,182	$249,041

Section 6. References

Watershed?	Reference
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☐	

PART II - NARRATIVE

Section 7. Abstract

Infectious hematopoietic necrosis is the most important viral pathogen in the culture of steelhead trout and commercial rainbow trout. There have been a number of vaccines formulated to immunize salmonids against IHNV, but many of the products were too expensive to produce on a large scale, were limited in their effectiveness, or concerns about reversion to virulence prevented their licensure and commercialization. The purpose of this proposal is to develop a method of delivery of IHNV immunogens using the bacterium, *Yersinia ruckeri*, as an adjuvant carrier which will simultaneously stimulate immunity to both *Y. ruckeri* and to IHNV. A current vaccine strain of *Y. ruckeri* will be used to express the IHNV G and N genes from the pTX101 plasmid. Utilizing a rainbow trout specific pathogen free model system, we will immunize broodstock with the formalin fixed *Yersinia* displaying the G and N proteins of IHNV and monitor passive transfer of maternal antibody into the egg and subsequently challenge the progeny from matings (control and vaccinated) to monitor protection against IHNV. Likewise, steelhead broodstock at Dworshak National Fish Hatchery will be monitored for antibodies against IHNV and subsequently immunized with the prepared vaccine. It is anticipated that eggs from matings of vaccinated broodstock will contain antibodies against IHNV and that the progeny will be protected against an early natural challenge of IHNV.

Section 8. Project description

a.   Technical and/or scientific background

Infectious Hematopoietic Necrosis Virus. Infectious hematopoietic necrosis virus (IHNV) is the most significant viral pathogen of salmonid fish in North America, causing epizootics in which up to 90% of fry and fingerlings in a population are lost (Winton, 1991). In addition to its presence in North America, IHNV has been documented in Asia (Sano, 1977) and has been introduced into Europe (Bovo, 1987). IHNV also poses a significant threat to endangered species where the losses of a few fish can be devastating.   The presence of IHNV in facilities which raise anadromous rainbow trout (steelhead) has been particularly troublesome. Not only does this pathogen cause significant mortality, but those juveniles which escape death can often be affected by scoliosis and lordosis (Busch, 1983). A released fish which is malformed due to IHNV exposure may be easily preyed upon in the wild, thus impacting management plans to repopulate streams with healthy fish.

As a member of the rhabdovirus family, IHNV is composed of a negative sense RNA genome coding for 6 proteins including a polymerase (L), two membrane proteins (M1 and M2), a nucleoprotein (N), a glycoprotein (G) and a nonvirion protein (NV) (McAllister, 1975; Kurath 1985ab). The glycoprotein (G) of IHNV has been recognized as the principal immunogen during infection, (Engelking 1989), although two recent studies have shown that the presence of the N protein along with G in immunization regimens has significantly increased the ability of a vaccine to protect fish (Oberg 1991; Anderson 1996a).

A number of vaccines have been developed to prevent IHNV infection. Among these are attenuated vaccines including the 100X pass Nan Scott Lake strain (Leong 1988) and the traditional formalin-killed virus (Nishimura 1985). The principal objection to the attenuated vaccine is that it may revert to virulence. An objection to the use of both of the latter vaccines is that production of antiviral vaccines in tissue culture is too expensive for an industry where the cost of vaccinating a single juvenile fish needs to approach one cent per fish to be cost effective.

Studies from our laboratories (LaPatra 1993; Ristow 1993) reveal that the G protein is the most frequently recognized viral antigen of IHNV on western blots using sera from survivors of multiple IHNV challenges. Furthermore, 82% of survivors show neutralizing antibodies against the virus, emphasizing how important the recognition of G is with respect to survival. Thus a number of researchers have produced subunit vaccines by cloning the genes for the G and N proteins into different expression systems. As with vaccination for rabies virus (Dietzchold 1987), the addition of the nucleoprotein to the IHNV glycoprotein vaccine appears to augment the efficacy (Oberg 1991; Anderson 1996a). Two groups used selected strains of *E. coli* which expressed the products as TrpE fusion proteins in the cytoplasm (Gilmore 1988ab; Leong 1993). Other expressions of IHNV G protein in the cytoplasm of *Aeromonas salmonicida* (Noonan 1995) and in the cytoplasm of *Y. ruckeri* (Estepa, 1994) have been isolated. Other approaches have included the cloning of the G gene into baculovirus (Koener 1990, Cain 1997) and the injection of naked DNA into juvenile trout in plasmid forms encoding both the G and N genes of the virus (Anderson 1996ab). Varying levels of protection have been achieved by all methods. To date, however, there is no licensed vaccine for IHNV.

Yersinia ruckeri. The gram negative bacterium *Yersinia ruckeri*, causative agent of enteric redmouth disease (ERM) or "Hagerman redmouth disease", is primarily a pathogen of rainbow trout, although it affects brown trout, brook trout, chinook and coho salmon. The principal symptom of infection with *Y. ruckeri* is a reddening of the mouth and throat of the trout caused by subcutaneous hemorrhages (Austin and Austin 1987). Additionally, there occurs darkening of the skin, erosion of the jaw, hemorrhaging at the base of the fins, and an accumulation of ascites within the body cavity. Twenty five years ago, ERM was the most important disease problem in the commercial aquaculture of trout (Hester 1973). Now, mainly due to an efficacious formalin killed bacterin, this economically important malady has been controlled (Austin and Austin 1987; Lillehaug 1995).

Studies of *Y. ruckeri* reveal a number of serotypes, certain subtypes of serotype 01 being virulent (Davies 1990, Davies 1991a). Virulence is partially correlated with resistance of the bacteria to the action of non-immune rainbow trout serum (Davies 1991a). Other investigators have implicated a heat sensitive factor, thought to be a lipid (Furones, 1990). Rainbow trout and brook trout are differentially resistant to certain clonal groups (isolates) of *Y. ruckeri* (Davies 1991a). Outer membrane profiles of *Y. ruckeri* extracted by the detergent Sarkosyl (Davies, 1991b) have revealed 5 unique outer membrane protein profiles. The band profiles are constant throughout the growth cycle with the exception that in several isolates the 39.5 kDa band is the most prominent when the cultures are in stationery phase. Many *Y. ruckeri* proteins have their equivalent proteins in *E. coli*. Davies speculates that the heat modifiable protein present in the protein profiles corresponds to the *E. coli* OmpA protein (Davies 1991b) and that the *Y. ruckeri* peptidoglycan associated proteins (PAP) (approximate molecular weight depending on the isolate of 36.5 to 40.5kDa) are the equivalent of the *E. coli* porin which forms hydrophilic pores through the outer membrane (OM).

Bacterial Surface Expression Systems of Gram Negative Bacteria. Because one of the objectives of this work involves the expression of the G and N proteins of IHNV on the outer membrane of *Y. ruckeri*, a gram negative bacterium, we will briefly review some of the more recent work in this area. Recently, Georgiou et al. (1997) have authored a review covering many of the known surface expression systems. Strategies for bacterial surface expression are numerous and have resulted in a number of novel applications including vaccines, bulk absorbents, catalysts, reagents for screening peptide libraries and solid phase diagnostics (Georgiou 1997).

For any surface expression strategy to work, the physical structure of the bacteria must be taken into account The cell wall of a gram negative bacteria consists of an inner membrane, a peptidoglycan constituting the cell wall, and an outer membrane. In order for material to be expressed on the surface, all three barriers must be crossed. The most successful strategies used for surface expression have used *E. coli* and have fused peptides into the outer membrane proteins (OMP systems) and to the major component of the *E. coli* flagellum (Flic; FLITRX system) (Lu 1995; Colas 1996). *E. coli* has been used for the expression of the peptides of the foot-and-mouth disease virus (Agterberg 1990c); beta-lactamase (Kornacker and Pugsley 1990; Francisco et al., 1992); epitopes of the preS2 region of the Hepatitis B virus (Charbit et al. 1987); recombinant antibodies (Fuchs 1991; Francisco 1993); and a cellulase (Francisco 1993). The Lpp-Omp A system is a modification of these, in which the heterologous protein is fused to the signal sequence of the major *E. coli* lipoprotein followed by the membrane spanning region of the Omp A. (Stathopoulos, 1996). This system has resulted in the expression of proteins up to 54kDa. Other prominent expression systems have included the display of antibodies (Su, 1992) and an 18 residue epitope of gp41 of HIV-1 (Wong, 1996) on the surface of *Salmonella typhimurium*. Additional surface expression systems have utilized *Shigella* (Suzuki, 1995)*, Klebsiella* (Kornacker, 1990)*, and Neisseria* (Salmond 1990) species.

Relationship of vaccination to Reproductive efficiencies. - There does not exist a great deal of literature on the effect of vaccinations on the quality of the offspring. The most efficacious vaccines are the bacterins, particularly for *Y. ruckeri* and *Aeromonas salmonicida* (Furunculosis) and for *Vibrios*. At the present time valuable broodstock such as Atlantic salmon are being vaccinated. There have been some problems with adjuvanted vaccines for Atlantic salmon, particularly those delivered in an adjuvanted oil base. When a trivalent vaccine (vibriosis, cold water vibriosis and furnculosis) in oil was administered by injection, there was a dramatic reduction in growth by 23% versus non-vaccinated controls and controls receiving the preparation enhanced with glucan (Mydtlyng and Lillehaug, 1998).

Only recently has it come to light that antibody can be passed from mother to fry in fish. The studies of Castillo et al. (1993) and Avatalion and Mor (1990) suggest that in immunized female tilapia, the ovaries and eggs contain antibody. The studies of Oshima et al. (1996) also indicate that there can be successful transfer of immunity against IHNV from immunized female trout to their offspring. When female broodstock were injected with a recombinantly produced peptide containing amino acids 31 to 310 of the glycoprotein, their eggs contained antibody to the virus. The mortality of the fry against challenge with IHNV was monitored. Survival was highest in the small fish when challenge occurred at 7 days post hatch, but immunity sometimes persisted 25 days.

b.   Rationale and significance to Regional Programs

This project has direct application to the increase in numbers of steelhead and other salmonids and the preservation of their health.

A national need has been identified for more efficacious vaccines for aquaculture (Broussard 1992; Leong 1993). IHNV has impacted both private and public aquaculture affecting Atlantic salmon in netpen culture, domestic trout hatcheries, and national and state hatcheries. Additionally, IHNV is an international problem with outbreaks in Japan, Italy, France, and Germany. Within intrastate and international commerce, trout eggs are shipped worldwide, and thus control of the virus is paramount. A formalin-killed efficacious bivalent vaccine which is produced as the result of expressing the G and N proteins of IHNV on *Y. ruckeri* will be not only environmentally safe but will immunize fish at an affordable cost for both public and private aquaculture operations.

IHNV has long been the scourge of the public hatcheries, destroying a significant number of steelhead fingerlings in the Pacific Northwest. The overall goal of this program is to immunize steelhead and trout against IHNV utilizing a recombinantly produced a killed preparation in which the carrier bacterium is *Y. ruckeri*, the agent of enteric redmouth disease, acts as an adjuvant and displays the G and/or N proteins on its surface. The goal is to immunize both broodstock directly and progeny passively or direcctly with this preparation and to challenge the immunized progeny. Such a bivalent vaccine will immunize fish to both pathogens at once. *Y. ruckeri*, already shown to be an effective vaccine, will act as an adjuvant to potentiate immunity against IHNV. The result will be more effective management against IHNV.

c.   Relationships to other projects

As a member of the UI/WSU Fish Reproduction Program, my laboratory will interact with the projects proposed by J. Cloud (Intracytoplasmic Sperm Injection: Genetic Retrieval from Single Sperm), J. Nagler (Endocrine Control of Ovarian Development in Salmonids), G. Thorgaard (Analyzing Genetic and Behavioral Changes During Salmonid Domestication), G. Schelling (Induction of Precocious Sexual Maturity and Enhanced Egg Production in Fish), and R. Ingermann (Enhancement of Salmonid Gemete Quality by Manipulation of Intracellular ATP).

d.   Project history (for ongoing projects)

Not applicable.

e.   Proposal objectives

The overall project objective is to construct a cost-effective, environmentally safe vaccine which has efficacy to proiect both fry and broodstock against both infectious hematopoietic necrosis (IHNV) and *Y. ruckeri*.   The overall hypothesis of this proposal is that this vaccine will immunize both domestic trout and wild steelhead against both *Y. ruckeri* and IHNV. It is also hypothesized that the reproductive efficiency of these animals will not be compromised and that the female fish will pass antibody on to her offspring through the egg such that the fry will be immune to IHNV and *Y. ruckeri*.

The specific subobjectives are to:
(1) Express the G and N proteins of IHNV separately on the surface of *Y. ruckeri*.
(2) Produce a killed bacterin, which is an effective bivalent vaccine.
(3) Test the vaccine for efficacy by immersion and/or injection immunizations followed by
        challenges with IHNV and *Y. ruckeri* and evaluation of the immune response.
(4) Examine the reproductive efficiencies of fish have been vaccinated by the bivalent vaccine and
        the eggs for the evidence of transfer of maternal antibody.
(5) Challenge vaccinated domestic trout broodstock (only as a model) with virulent IHNV to
        assess whether the egg-associated passive immunity was efficacious.
(6) Test the model vaccine in a public hatchery, Dworshak National Fish Hatchery.

f. **Methods**

OBJECTIVE I. Express the G and N genes of IHNV on the surface of Y. ruckeri.

The IHNV G and N proteins will be expressed on the surface of the *Y. ruckeri* using the expression plasmid pTX101 (Francisco 1992) which has been previously shown to express beta-lactamase on the surface of transformed *E. coli* JM109 (Francisco, 1992). The plasmid pTX101 has been provided to us by the laboratories of Dr. George Georgiou. Dr. Jo-Ann Leong, Oregon State University, has kindly agreed to give us the N gene (Oberg 1991) and has already shared the G gene both in the prokaryotic form (Gilmore 1988a) and in the eukaryotic form (Koener 1987; Koener 1990). Oregon State University has patented the G and N genes and they are available on a nonexclusive basis. As described in our review of the current literature, surface expression with pTX101 is accomplished utilizing the signal sequence of the *E. coli* lipoprotein (Lpp) followed by a gene fragment for the OmpA membrane-spanning section. Upon transformation of the bacterial culture, we will follow the appearance of the foreign protein on the surface of the bacterium by flow cytometry using the FACSCalibur flow microfluorimeter and monoclonal antibodies made to the G and N proteins of IHNV made in Dr. Ristow's lab (Ristow 1991). We plan to express the G and N proteins of IHNV on the surface of *Yersinia ruckeri* by replacing the beta-lactamase gene in pTX101 with the gene for both the G or N proteins of IHNV in separate constructs.

Construction of Expression Plasmids for G and N. - The G gene and N genes of IHNV in plasmids are patented by Oregon State University and are available on a nonexclusive basis for research purposes to anyone interested in using the technology commercially, as mentioned previously. PCR primers will be designed based on the 5' and 3' sequence of the G and N genes. An additional facet of primer design will be the addition of an Eco RI endonuclease site on the 5' primer and a Bam HI endonuclease site on the 3' primer for directional cloning into pTX101. The primers will be designed to place the coding sequence of G and N in frame with the Lpp-OmpA reading frame. pTX101 will be digested with Eco RI and Bam HI to release the gene fragment coding for beta-lactamase. The digested plasmid will then be purified from a low-melting temperature agarose gel and mixed in a ligation reaction with the PCR product from the G or N gene. The ligation reaction will be transformed into *E. coli* and clones selected by antibiotic resistance. Colonies will be screened for the correct insert by restriction enzyme digest. Positive clones will be partially sequenced from both the 5' and 3' ends of the insert, using primers designed from the known sequence of pTX101. The resulting sequences will be analyzed for correct reading frame. The clones intended for use will be fully sequenced to assure there has not been any alteration of the G or N gene.

Expression of the G and N genes. –*Y. ruckeri* and *E. coli* will be transformed with either the unaltered pTX101 or the new expression plasmid containing the G gene (pTXG) or N gene (pTXN). *E. coli* transformed with pTX101 and *E. coli* transformed with pTXG or pTXN will serve as the controls for protein expression. *Y. ruckeri* transformed with pTX101 will serve as the control for protein expression in *Yersinia*. Expression will be determined by surface immunofluorescence using a FACSCalibur flow microfluorimeter and commercially available antibodies to beta-lactamase and monoclonal antibodies we have produced to IHNV G and N proteins (Ristow 1991) and antibodies recognizing neutralizing epitopes of the G protein, a gift of Dr. James Winton, National Biological Survey, Seattle (Huang 1994). In addition, protein expression will also be characterized using immunblot and immunohistochemistry. We will monitor the expression of the G and N proteins on the surface of *Y. ruckeri* using the FACSCalibur as the principal means of assaying for expression (Daugherty, 1997). Dr. Byrne has expertise in this area and in addition, has completed the course on the use of the FACSCalibur instrument at Becton-Dickinson. We have a library of monoclonal antibodies which define both the tertiary and linear epitopes of G and N proteins (Ristow 1991). In addition, Dr. Jim Winton has provided us with 5 additional neutralizing monoclonal antibodies which may define tertiary epitopes of the virus (Huang 1994). Thus it should be possible to note whether the monoclonal antibodies react with the epitopes of the G or N proteins as displayed on the surface of *Y. ruckeri* via the FACS method.(Daugherty, 1997).

Potential Problems and Corrective Actions. - The first potential difficulty is the compatibility of pTX101 to *Y. ruckeri*. Should we find that pTX101 does not express the beta-lactamase gene in *Y. ruckeri*, we have the remaining alternative of utilizing *E. coli* to express the G and N genes of IHNV. Although this would not produce a bivalent vaccine against *Y. ruckeri*, it would still have the advantage of the bacterial delivery system which may induce a stronger immune response than preparations of the virus or its subunits alone. A second pitfall would be the possibility of toxic expression of recombinant protein. If the

G or N proteins are overproduced in either bacterium, a recombinant clone may not be isolated because of the toxic effect. To overcome the possibility of overexpression in *Yersinia*, however, an alternative inducible promoter could be substituted for the lpp/lac promoter of pTX101. This would allow control over plasmid protein expression and allow recombinant *Yersinia* clones to be isolated.

       Results Expected. At the conclusion of Objective 1, plasmids pTXG and pTXN containing the G and N proteins of IHNV will have been isolated and their correct orientation and frame determined by sequencing. We will have determined whether pTX101 is compatible with *Y. ruckeri* based on expression of beta-lactamase. *Y. ruckeri* and *E. coli* will have been transformed pTXG and pTXN and the expression of the G and N proteins on their outer wall determined by surface immunofluorescence using a full array of monoclonal antibodies in flow cytometry.

OBJECTIVE II. Produce a two killed bacterins, one containing the G gene and one containing the N gene in Y. ruckeri

Method: At present, the *Y. ruckeri* bacterin at Clear Springs Foods is prepared in a fermentor and is fixed with 0.5% formalin to produce a monovalent vaccine. We plan to grow the recombinant bacteria in log phase and fix the bacteria at maximal expression of foreign surface proteins with formalin. The kinetics of recombinant protein expression will be determined by first identifying the log and stationery time phases for the bacteria and then sampling for protein expression. We will test for protein expression and the immunoreactivity of G and N using the available monoclonal antibodies to G and N and positive trout sera for IHNV. For *Yersinia* immunoreactivity, we will use commercially available antisera from MicroTek, Inc. Immunoreactivity will be tested in native and denatured immuoblots against pre-inactivation recombinant *Yersinia*, IHNV and *Yersinia*. Once we have tested the reactivity *in vitro*, a small number of fish will be innoculated with the formalin preparation to test their *in vivo* immunogenicity. Sera will be tested by immunoblot against IHNV and the vaccine preparations and neutralizing antibody titers to IHNV determined in procedures currently in use in our laboratories.

       Pitfalls and Limitations. - A potential problem with this objective is the use of formalin to inactivate the recombinant *Y. ruckeri* and the possible alterations in the immunogenicity of the G and N proteins displayed on the surface. If we find from our immunoblots, neutralization assays, or preliminary challenge data that the formalin inactivated G and N are not recognized by the trout, we will consider other methods of inactivation such as beta-propriolactone which has been successful in retaining the antigenicity of IHNV in the killed vaccine used in a recent trial (vaccine trial - IHNV workgroup 1998).

       Results Expected. - At the conclusion of Objective 2 , a formalin or beta-propiolactone inactivated recombinant vaccine preparation of *Y. ruckeri* expressing either the G or N proteins of IHNV will have been produced which shows immunogenicity in trout. Small batches of this material will have been prepared for testing and challenge at CSF.

OBJECTIVE III: Test the killed bacterin for efficacy in protecting rainbow trout fry from challenges of Y. ruckeri and IHNV.

Fish and Immunizations. -The following protocols will be used in the model system for immunizing juvenile domestic trout and juvenile steelhead and follow the standard protocols used by Dr. LaPatra at the CSF Research Facility. Domestic stocks will be obtained from the specific pathogen free fish grown in the research facility. At CSF prior to immunization and challenges, fish are held in 116 liter tanks at 15$^{\text{o}}$C in a single pass flow-through system. One gram trout will be immunized by intraperitoneal injection of 10-50 μg of bacterial protein or dipped in a standard dilution of the killed vaccine according to the protocol used at Clear Springs Foods to immunize against *Yersinia* described below. Because the killed recombinant vaccine will be derived from the CSF strain of *Yersinia,* which has been so effective in past immunization protocols, we prefer to use Dr. LaPatra's present immersion protocol because it is much more cost effective and less time consuming. Fish will be dipped in a 1:10 dilution of the vaccine for 60 seconds and removed to fresh water. Immersions will take place in which other groups of fish are dipped in various dilutions of the vaccine (to vary the concentration). Groups will be immunized by the N protein construct, the G protein construct, equal volumes of G and N construct, the standard CSF *Yersinia* bacterin, a media control, and another control group will be maintained without any manipulation. Housed in a separate area will be a final group of trout vaccinated with the 100X pass Nan Scott Lake virus (positive control

attenuated virus vaccinated group)(Cain 1996). The fish will be challenged after 4 weeks. At two weeks post-vaccination, 5 fish in each group will be euthanized. Their sera and lymphocytes will be harvested for in vitro immunologic characterization by neutralization assays and lymphocyte proliferation. Each experimental group will contain 125 fish. Replicate groups will be included such that one group is challenged with *Y. ruckeri* while the other is challenged with IHNV. Thus we can measure the efficacy of the bacterins with respect to both pathogens.

Preparation of Yersinia ruckeri for a static bath challenge. - *Y. ruckeri* (Isolate 007-82; Serotype 01) is removed from the -80°C freezer and struck for single colony isolation on a typticase soy agar. It is then grown for 48h at 20°C. The identity of the colonies is confirmed by plate agglutination with homologous polyvalent antisera. Ten colonies are picked from the plate with a loop and the material diluted in 3 ml trypticase soy media. Two plates of trypticase soy agar are swabbed with the diluted bacteria to produce a lawn for each 10 treatment groups to be challenged. The agar plates are incubated for 48h at 20°C. On the day of the challenge, bacteria are harvested from the plates in 0.9% saline. A stock cell suspension of *Y. ruckeri* is adjusted to 50% transmittance at 640 nm, diluted 1:10 and is used as the challenge stock.

Preparation of Virus for Challenges. A low passage of the 220-90 isolate (LaPatra, 1991) of IHNV is propagated in chinook salmon embryo cells at 18°C. Aliquots of the virus are frozen at -80°C preparatory to challenge. Virus is plaqued on EPC cells prior to the challenge in order to determine the number of plaque-forming units per mL.

Virus challenge. Groups of 25 to 50 trout will be challenged by exposure to either 10^4 or 10^5 units per mL of the 220-90 isolate of IHNV (LaPatra et al, 1991) in a total volume of water that is ten times the weight of the fish for 1 hour with aeration. After challenge the fish are removed to separate 22L aquaria receiving constant temperature (15°C) ultraviolet disinfected single pass spring water. The fish are fed ad libitum (4X daily) and monitored for mortality for 21 days.

Bacterial challenge. Certified specific pathogen free (SPF) rainbow trout fingerlings will be randomly drawn from a single uniform graded production lot with an average individual weight of 5 grams, or a sample count of 90.8 fish per pound 48 hours prior to the day of challenge, and taken off feed the day prior to and the day of challenge. In order to simulate the natural course of disease, challenges will be done at a standard bath loading density of 100 grams of fish per 1,000 ml of water containing the *Yersinia* suspension. Fish will be challenged for 60 minutes in a static bath with supplemental oxygention.

Statistics. Cumulative percent mortality between variously immunized groups and the controls are determined by ANOVA on transformed (asin /p) data (Snedocor and Cochran, 1967).

Results Expected. - At the end of Objective 3, we will have determined the efficacy of our recombinant *Yersinia* as a bacterial delivery system for IHNV proteins G and N. Its efficacy will have been determined by in vitro immunoassays and pathogen challenge. We will also have evaluated the effectiveness of combining G and N preparations as well as any potential adjuvant effect that bacterial presentation may have.

4. OBJECTIVE IV. *Examine the reproductive efficiencies of fish which have been vaccinated with the bivalent vaccines and test their eggs for evidence of transfer of maternal antibody.*

Fish and Immunizations: Broodstock females (16 months old, mean weight 2 kg) will be maintained in the Clear Springs Foods Research Facility on single pass specific pathogen free water. Each fish will be individually tagged and baseline immunity to IHNV assessed. The presence of antibodies in the serum of the females will be assessed by virus neutralization procedures (LaPatra et al. 1993) on serum samples taken from the fish 3 weeks prior to immunization. Females will be injected with the killed recombinant vaccine 4 times at three week intervals. Nonlethal serum samples will also be collected at 8, 16, and 14 weeks after the first immunization. The serum samples will be analyzed for the presence of neutralizing antibody and by the ELISA for total antibody against IHNV proteins (Ristow et al 1993). Upon spawning, a portion of the eggs from G and N vaccinated, mock vaccinated (with the *Yersinia* vaccine alone) and the untreated fish will be removed and a portion of the eggs used to produce an egg extract (Oshima, 1996). The egg extract will be analyzed for presence of anti IHNV antibodies (Oshima 1996). Fecundities, egg eye-up and hatching rates will also be determined for each treatment. Eggs from recombinant vaccinated, *Yersinia* bacterin vaccinated and mock vaccinated broodstock will be included in the analysis.

OBJECTIVE V. *Challenge the progeny (hatched fry) of the vaccinated female brood fish with IHNV to determine whether the presence of maternal antibody is efficacious, protecting the fry from a challenge of virulent IHNV.*

Fish and Immunizations. The present protocols follow the standard protocols used by Dr. LaPatra at the CSF Research Facility. Fish used for this objective will be the progeny of the female broodstock fish noted in the previous objective. The fry will be challenged when they reach 0.5 - 2 gram size using the protocol for challenge presented under the previous objective. Prior to immunization and challenges, fish are held in 19 liter tanks at 15^oC in a single pass flow-through system.

Preparation of Yersinia ruckeri for a static bath challenge. Preparation and challenge with *Y. ruckeri* will be as previously stated under methods in Objective III.

Preparation of Virus for Challenges. Preparation of virulent IHNV virus will be as previously stated under objective III.

Virus challenge. Fish will be challenged and cared for as described under the methods for objective III.

Bacterial challenge. Bacterial challenges will take place as stated under objective

Statistics. Cumulative percent mortality between variously immunized groups and the controls are determined by ANOVA on transformed (asin /p) data (Snedocor and Cochran, 1967).

Results Expected At the end of Objective 5, we will have determined the efficacy of the vaccines to protect the progeny by transfer of maternal antibodies via the egg. In other words, we will prove or disprove the hypothesis that properly immunized fish can confer maternal antibody (by passive transfer through the egg) to their offspring. It is anticipated that the fry from mother fish vaccinated with the G plus N *Yersinia* blended recombinant protein product will have a greater survival than those fry out of mothers vaccinated with *Yersinia* alone or mock-vaccinated with PBS.

OBJECTIVE VI. Vaccinate steelhead broodstock at Dworshak National Fish Hatchery and determine the effect on eggs and progeny. [Modifications of protocols in objective 5 for conducting experiments on broodstock and juveniles at Dworshak National Fish Hatchery]

The killed and recombinant vaccine will be tested at Dworshak National Fish Hatchery by permission of Mr. Bill Miller, Manager of the Dworshak-Ahsahka Complex, and Mr. Howard Burge, and with the collaboration of the personnel in the Dworshak Fish Health Laboratory. At present, it is envisioned that we will first determine the titers of individual broodstock to IHNV by both ELISA and complement neutralization (Ristow 1993; LaPatra 1993) and then immunize the broodstock. Matings will be made between the sperm and eggs of broodstock according to the prevailing management plans within the hatchery. A portion of the fertilized eggs resulting from each mating (from controls and vaccinated individuals) will be cracked open and the contents assayed for the passive transfer of antibody (Oshima 1996). Progeny of matings will be placed in raceways in floating enclosures for exposure to natural challenges of IHNV.

Juveniles will also be vaccinated by either immersion or by i.p. injection, contained in floating cages and allowed exposure to natural challenge by IHNV within the hatchery. [Challenges with *Y. ruckeri* will <u>not</u> be conducted at Dworshak because there are no appropriate facilities for controlled challenge.]

g.   Facilities and equipment

Research will be performed in the laboratories of the investigators in the recently constructed Animal Science Laboratory Building. The Department of Animal Sciences was recently awarded an equipment grant for the purchase of a Becton-Dickinson FACsCalibur (Katherine M. Byrne, PI) and an ABI 377XL DNA Sequencer (Sandra Ristow, PI). Common equipment for radioimmuoassay includes geiger counters, fraction collectors, columns and special chemical hoods for containment of radionuclides. Counting equipment available includes an IsoData 20-20 gamma counter interfaced with a GE computer and an ICN Scintillation counter. Centrifugation equipment consists of a Sorvall OTD65 ultracentrifuge equipped with AH627 swing bucket and fixed angle rotors, a Beckman GPR tabletop centrifuge and International centrifuges PR-1 and PR-6. A new

Beckman L7-80 ultracentrifuge with SW 28, SW50.1 and SW41 rotors are also common equipment. Common equipment shared among labs includes Sorvall High and Low Speed centrifuges, a Sorvall preparative ultracentrifuge, a Scotsman ice machine, a Virtis Model II lyophilizer, Sartorius analytical balances, a Perkin-Elmer fluorimeter with recorder, a Perkin-Elmer gas chromatograph with recorder, and a liquid scintillation counter. Other equipment and resources available on campus include the Electron Micropscope Center which has an Amray scanning and Hitachi 600 transmission electron microscopes as well as a BioRad MRC-1024 Laser Scanning Confocal Microscope with 488, 568, and 647 excitation. Also available on campus are the WSU VADAMS computer lab, the Laboratory for Biotechnology and Bioanalysis for peptide sequencing and synthesis, DNA synthesis and amino acid analysis, and the Washington Animal Disease Diagnostic Laboratory, which offers complete histology services.

Dr. Sandra Ristow

Dr. Ristow's laboratory consists of 1000 square feet of space in the new animal laboratory building and 120 square feet of office space. The lab is equipped with Baker and CCI tissue culture hoods, two Fisher low temperature incubators for producing virus, Forma Stericult incubator for hybridoma production, a small Perkin Elmer PCR 2400, a new BioRad CHEF III Pulse Field Electrophoresis, Savant DNA110 SpeedVac, BioRad protein gel electrophoreses and power supplies, Hoefer gel electrophoresis and western blot device, an EC 4000P power source, gel dryer, a Skatron cell harvester, a TA60 biological freezer for hybridoma cells, and a Forma model 8459 low temperature freezer. There is an IGEN Origen immunoassay system. A new DNA sequencer, a Perkin Elmer ABI Prism 377XL, acquired with USDA funding (S. Ristow, PI), is available for sequencing needs in the LBB-1 central facility.

Dr. Katherine M. Byrne

Dr. Byrne has been assigned approximately 1000 square feet of laboratory space in the new animal sciences laboratory building, and 120 square feet of office space. Her laboratory is equipped with A FACsCalibur fluorescence activated cell sorter, chemical fume hood, a class II tissue culture hood, a Forma stacked CO_2 incubator, Leica inverted phase-contrast microscope and a dual fluorescent scope. Immunohistochemistry is performed using the MicroProbe staining apparatus and slide incubator. Other items include a Sorvall table-top centrifuge, Hoefer protein electrophoresis vertical gel units and western transfer apparatus, a Juoan refrigerated microfuge, and a Perkin-Elmer spectrophotometer with microvolume cuvettes. Hybridization, both Southern and Northern, can follow electrophoresis using the Hoefer semi-dry transfer units and Robbins-Scientific hybridization oven. A Perkin-Elmer 2400 thermocycler is available for PCR reactions. Other equipment in Dr. Byrne's laboratory includes a survey meter for radioactive work, a refrigerated water bath, a low temperature incubator, a microwave, a pH meter, vortexes, hot plates, multiple heated water baths, refrigerators, a -20 freezer, and a -70 freezer.

Dr. Scott LaPatra

All *in-vivo* work for evaluation of the vaccine in the trout SPF model system will be conducted at Clear Springs Foods (CSF), Inc., Research and Development Complex (Buhl, Idaho). IHNV susceptibility challenges will be performed in the Specific Pathogen Infected (SPI) laboratory. The SPI laboratory is provided with constant temperature (15°C), ultra-violet (UV) disinfected spring water. Current aquaria available consist of five 379 L, ten 114 L, and seventy 19 L tanks. All water discharged from the SPI laboratory is disinfected with an automatic iodine drip pump and held in retention tanks in series before flowing into a leach field. There is also additional aquarium space for noninfectious investigation in the General Laboratory (GL) including nine 1136 L tanks and fifty 379 L tanks. All fish used in experiments will be obtained from CSF broodstock and incubated, hatched, and reared in the Specific Pathogen Free (SPF) laboratory. There is also a dry laboratory with adequate space and equipped with media preparation laboratory, microfuge, preparative centrifuges, ultracold freezer, fume hood, biohazard safety hood, cold cabinet, fermentor, UV disinfected transfer rooms for tissue culture, inverted microscopes, and incubators.

h.   Budget

Personnel: Three-fourths of a month of summer salary for the PI is requested. It is estimated that the time of 1.5 technical personnel will be required for the project. One of the persons will be Mr. Richard Dixon who is the chief technician in Dr. Ristow's laboratory. One half of his salary is paid by state funds, thus one-half salary is

requested for him. In addition, we are requesting $2,000 in student timeslip funds. Students clean the laboratory, run errands to the central storehouse, do reference work in the library and clean and sterilize labware.

Benefits are 26% base salary for faculty, 29% base salary for staff and 9% for timeslip workers.

Supplies include bacterial media, restriction enzymes, cloning kits, PCR supplies, protein blot reagents, cell culture media, fetal calf serum, immunohistochemicals, molecular biology supplies, chemicals, cell culture plastic, plastics for molecular biology, bacteriological and media glassware and autoradiography supplies.

$3000 is included for instrument repair and maintenance (refrigerators, freezers, pipetors, incubators).

Funding is requested for travel to the AFS Fish Health Section Meeting.

Indirect costs are calculated at 45% of direct costs.

Two items of equipment are requested. The Stratagene PCR is requested because other ongoing projects in both Dr. Ristow's and Dr. Byrne's laboratories are putting great pressure on the use of the two small 24 well machines in the Ristow and Byrne laboratories. In addition, there is a need for a reliable documentation system for gels in the Ristow laboratory, thus the request for the Stratagene system.

Funds are requested to support both the administrative core of the Center for Reproductive Biology at WSU and the Aquaculture Core Facility, which will be utilized in subsequent years of this proposal.

Costs have been projected to rise at 5% per year.

A breakdown of costs on the two items of equipment is found below:

Stratagene 400884 Robocycler PCR, w/hot top assembly 120V	$7,895
10% tax and shipping	$790
Subtotal	$8685

Stratagene Eagle Eye II™ Documentation System includes:	
Standard CCD Camera/Pentium System, 120V	$11,795
Eagle Eye Darkroom Cabinet, 120V	$2,995
Standard Format Thermal Printer, 100/120V	$2,795
Subtotal	$17,585
10% tax and shipping cost	$1759
Subtotal for documentation system	$19344

Section 9. Key personnel

Principal Investigator: Sandra S. Ristow

Present Position: Professor of Animal Sciences, Washington State University, Pullman WA 99164-6351

Education:

University of Minnesota, Minneapolis, MN, Ph.D., 1972, Biochemistry
Wisconsin State University, Eau Claire, B.S., 1963, Chemistry
University of Minnesota Medical School, Mpls., Postdoctoral in immunology, 1972-1976

Recent Employment History

Professor and Animal Scientist - Washington State University - Department of Animal Sciences - July 1997

Associate Professor and Associate Animal Scientist - Washington State University - Department of Animal Sciences - 1992 to 1997

Associate Investigator - Oregon State University - Marine/Freshwater Biomedical Center - October 1995 to Present

Assistant Professor and Assistant Animal Scientist - Washington State University - July 1988 to July 1992

Relevant Publications:

Ristow SS, J de Avila, SE La Patra, K Lauda. Detection and characterization of rainbow trout antibody against infectious hematopoietic necrosis virus. Diseases of Aquatic Organisms 15:109-114. 1993.

Helmick CM, JF Bailey, SE LaPatra, SS Ristow. Esophagus/cardiac stomach region: site of attachment and internalization of infectious hematopoietic necrosis virus in challenged juvenile rainbow trout (*O. mykiss*) and coho salmon (*O. kisutch*). Diseases of Aquatic Organisms 23(3):188-199. 1995.

Cain KD, SE LaPatra, T Baldwin, B Shewmaker, J Jones, SS Ristow. Characterization of mucosal immunity in rainbow trout (*Oncorhynchus mykiss*) challenged with infectious hematopoietic necrosis virus: identification of antiviral activity. Diseases of Aquatic Organisms 27(3):161-172. 1996.

Cain KD, KM Byrne, Al Brassfield, SE LaPatra and SS Ristow. Temperature dependent characteristics of a recombinant infectious necrosis virus glycoprotein produced in insect cells. [In press, 1998, in Diseases of Aquatic Organisms]

Cain KD, SE Lapatra, B Shewmaker, J Jones, KM Byrne and SS Ristow. Immunogenicity of a recombinant infectious hematopoietic necrosis virus glycoprotein produced in insect cells. [In press, 1998, in Diseases of Aquatic Organisms]

Co-Investigator: Katherine M. Byrne

Present position: Assistant Professor, Department of Animal Sciences, Washington State University

Education:

DVM, University of Missouri, 1986
Ph.D. Veterinary Microbiology, Louisiana State University, 1992

Recent Employment History:

Assistant Professor, Department of Animal Sciences, Washington State University, Pullman WA 1996-present

Assistant Professor, Department of Veterinary Microbiology and Pathology, Washington State University, 1992-96

Relevant Publications:

Byrne KM, Davis WC, Holmes M, Brassfield AL, McGuire TC. Cytokine RNA expression in an equine CD4+ subset differentiated by expression of a novel 46 kDa surface protein. Veterinary Immunology and Immunopathology 56:191-204, 1997.

Byrne KM, Cheng X, Vierck J, Erickson S, Greene EA, Duckett S, Dodson MV. Use of a 96-well plate reader to evaluate proliferation of equine satellite cell clones in vitro. Methods in Cell Science.19:311-314, 1998.

Tschetter J, Byrne KM, Perryman L, Hines MT, McGuire TC. Control of equine infectious anemia virus is not dependent on ADCC mediating antibodies. Virology, 230:275-280, 1997.

Cain KD, KM Byrne, Al Brassfield, SE LaPatra and SS Ristow. Temperature dependent characteristics of a recombinant infectious necrosis virus glycoprotein produced in insect cells. [In press, 1998, in Diseases of Aquatic Organisms]

Cain KD, SE Lapatra, B Shewmaker, J Jones, KM Byrne and SS Ristow. Immunogenicity of a recombinant infectious hematopoietic necrosis virus glycoprotein produced in insect cells. [In press, 1998, in Diseases of Aquatic Organisms]

Co-Investigator: Scott Edward LaPatra

Present position: Director of Research and Development, Clear Springs Foods, Buhl, Idaho

Education:
Ph.D. Oregon State University, 1989, Microbiology.
B.S. Oregon State University, 1979, Biology.

Recent Employment History:

Director of Research and Development, Clear Springs Foods, Inc., 1998-present
Research Scientist, Clear Springs Foods, Inc., 1990-1998
Associate Fish Pathologist/Virologist, Oregon Department of Fish & Wildlife, Oregon State University, 1987-1990

Relevant Publications:

Anderson, E.D., D.V. Mourich, S. Fahrenkrug, S.E. LaPatra, J. Shepherd, and J.C. Leong. 1996. Genetic immunization of rainbow trout (*Oncorhynchus mykiss*) against infectious hematopoietic necrosis virus. Molecular Marine Biology and Biotechnology 5(2):114-122.

Cain, K.D., S.E. LaPatra, T. Baldwin, B. Shewmaker, G.R. Jones, and S.S. Ristow. 1996. Characterization of mucosal immunity in rainbow trout (*Oncorhynchus mykiss*) challenged with infectious hematopoietic necrosis virus; identification of antiviral activity. Diseases of Aquatic Organisms 27:161-172.

Traxler, G.S., J.R. Roome, K.A. Lauda, and S.E. LaPatra. 1997. The appearance of infectious hematopoietic necrosis virus (IHNV) and neutralizing antibodies in sockeye salmon (*Oncorhynchus nerka*) during their migration and maturation period. Diseases of Aquatic Organisms 28:31-38.

LaPatra, S.E. 1998. Factors affecting pathogenicity of infectious hematopoietic necrosis virus for salmonid fish. Journal of Aquatic Animal Health 10:121-131.

LaPatra, S.E., K.A. Lauda, G.R. Jones, W.S. Shewmaker, and C.J. Bayne. 1998. Resistance to IHN virus infection in rainbow trout *Oncorhynchus mykiss* is increased by glucan while subsequent production of serum neutralization activity is decreased. Fish and Shellfish Immunology 8:435-446.

Section 10. Information/technology transfer

This project should yield a characterized vaccine for IHNV and *Yersinia ruckeri* which will be made available. In addition, papers resulting from these investigations will be published in refereed nationally recognized journals.

Congratulations!